

Heparin-binding epidermal growth factor–like growth factor promotes transcoelomic metastasis in ovarian cancer through epithelial-mesenchymal transition

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Abstract

Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is involved in several biological processes including cell adhesion, invasion, and angiogenesis. HB-EGF also plays a pivotal role in the progression of ovarian cancer. To investigate the significance of HB-EGF in peritoneal dissemination, we examined the roles of HB-EGF in cell adhesion, invasion, and angiogenesis in ovarian cancer. Through the suppression of focal adhesion kinase and EGF receptor activation, cell adhesive properties mediated by integrin β_1 were diminished by the inhibition of HB-EGF expression. The reduction of HB-EGF expression attenuated the chemotactic invasive ability and the expression of matrix metalloprotease (MMP)-2 and vascular endothelial growth factor (VEGF), leading to the inhibition of cell invasion and angiogenesis. Suppression of the Snail family, which regulates the epithelial-mesenchymal transition, blocked the cell adhesion properties on extracellular matrices, the chemotactic invasive ability, and the expression of MMP9 and VEGF through the reduction of HB-EGF expression. The volume of tumor burden in the peritoneal cavity was dependent on the expression of HB-EGF. According to these results, HB-EGF contributes to cell adhesion, invasion, and angiogenesis, which are integral to transcoelomic metastasis in ovarian

cancer. CRM197, an inhibitor of HB-EGF, resulted in a significant decrease of tumor burden in peritoneal dissemination, accompanied with a reduction in both cellular spreading, when assayed on an extracellular matrix, and invasive ability, when assayed in a chemotaxis chamber, as well as decreased expression of MMP9 and VEGF. Thus, HB-EGF is a mutual validating target in the peritoneal dissemination of ovarian cancer, and CRM197 may be useful as a anticancer agent for advanced ovarian cancer. [Mol Cancer Ther 2008;7(10):3441–51]

Introduction

Ovarian cancer, which is one of the most highly malignant cancers in women, is characterized by an extremely poor prognosis due to the wide extension of ovarian cancer cells into the peritoneal cavity. Approximately 70% of patients reveal late-stage ovarian cancer at the time of original diagnosis (1). Moreover, all established therapies have indicated a poor efficiency in the late stage of the disease (2). Although chemotherapies have been further optimized in the last decade, the mortality due to ovarian cancer remains unchanged. Accordingly, new therapeutic strategies for advanced ovarian cancer treatment are urgently required.

Metastasis from epithelial ovarian cancer can occur via the transcoelomic, hematogenous, or lymphatic route. Of these metastatic routes, transcoelomic metastasis is the most common and is responsible for the greatest morbidity and mortality in ovarian cancer (3). The metastatic dissemination generally occurs in four steps: (a) survival of cancer cells in the peritoneal fluid after detaching from the primary tumor, (b) adhesion of cancer cells to the peritoneum, (c) motility and invasion of cancer cells into the peritoneum, and (d) tumor formation through angiogenesis induced by cancer cells. Ascitic fluid in ovarian cancer contains several substances such as lysophosphatidic acid, heparin-binding epidermal growth factor–like growth factor (HB-EGF), and vascular endothelial growth factor (VEGF), which contribute to the survival of cancer cells (4–6). Ovarian cancer cells mainly adhere to mesothelial cells via integrin β_1 interactions with extracellular matrices (7). Once attached to the surface of the peritoneal membrane, the metastatic cells proliferate and penetrate into the subjacent mesothelium. Matrix metalloprotease (MMP)-9 and VEGF are major contributors to the process of stromal invasion and angiogenesis in ovarian cancer (8, 9). Epithelial-mesenchymal transition (EMT) has been associated with the development of anoikis and resistance to apoptosis in various cancer cell lines, suggesting that it confers a survival advantage to metastatic cells (10). Up-regulation of the Snail transcriptional repressor family has

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been implicated in the metastasis or progression of ovarian cancer, suggesting that these molecules contribute to peritoneal dissemination (11). By identifying the key molecule involved in the steps of transcoelomic metastasis in ovarian cancer cells, it would allow us to advance the development of molecularly targeted therapy for advanced ovarian cancer.

HB-EGF, which belongs to the family of EGF receptor (EGFR) ligands, is initially synthesized as a transmembrane protein, similar to other members of the EGF family of growth factors (12, 13). The membrane-anchored form of HB-EGF (proHB-EGF) is cleaved at the cell surface by a protease to yield the soluble form of HB-EGF (sHB-EGF) via a mechanism known as ectodomain shedding (14). HB-EGF, which is a potent mitogen and chemoattractant for a number of different cell types, can contribute to cell adhesion, cell motility, and angiogenesis (15). Recently, increasing evidence is accumulating for the critical role of HB-EGF in ovarian cancer cell growth and tumor progression, indicating that HB-EGF is a promising target for ovarian cancer (6, 16, 17).

The aim of this study is to investigate the validation of HB-EGF as a therapeutic target for advanced ovarian cancer. We found the relevance of HB-EGF to cell behaviors including cell adhesion, invasion, and angiogenesis in ovarian cancer. In addition, human mesothelial cells lying in the peritoneum, where cancer cells had invaded, activated the production of HB-EGF. Accordingly, HB-EGF regulates the behavior of ovarian cancer cells in the peritoneal cavity. CRM197, a specific inhibitor for HB-EGF, prohibited cell adhesion, invasion, and angiogenesis in ovarian cancer, indicating that CRM197 may be a promising therapeutic agent for advanced ovarian cancer.

Materials and Methods

Reagents and Antibodies

Diphtheria toxin (DT) and CRM197 were prepared as described previously (18). To administer CRM197 to mice, lipopolysaccharide-like materials contaminating the CRM197 preparations were removed using Detoxi Gel (Pierce Biotechnology). Recombinant human HB-EGF, transforming growth factor (TGF)- β , and endothelin-1 were purchased from R&D Systems, Inc. GM6001, PD153053, and PD98056 were purchased from Calbiochem. GM6001 is a metalloprotease inhibitor used to inhibit the ectodomain shedding of HB-EGF. PD153053 or PD98056 is a mitogen-activated protein kinase kinase (MEK) or EGFR inhibitor. Human fibronectin, collagen type I, and collagen type III were purchased from BD Biosciences. Mouse monoclonal anti-integrin β_1 antibody, which functions as an adhesion-blocking agent for extracellular matrices, was obtained from Chemicon International, Inc. Polyclonal rabbit anti-EGFR, anti-extracellular signal-regulated kinase (ERK), anti-VEGF, and anti-E-cadherin antibodies and mouse monoclonal antibody against MMP9 were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-phospho-ERK, anti-phosphotyrosine, anti-focal adhesion

kinase (FAK), and anti-integrin-linked kinase antibodies were obtained from Upstate Biotechnology, Inc. Mouse monoclonal anti- β -actin antibody was purchased from Sigma. Peroxidase-conjugated sheep anti-mouse IgG, peroxidase-conjugated goat anti-rabbit IgG, and peroxidase-conjugated sheep anti-goat IgG were obtained from Amersham Corp. and Zymed, respectively.

Plasmid Construction and Transfection

Construction of plasmids encoding human proHB-EGF cDNA inserted into the eukaryotic expression vector pRc/CMV (Invitrogen) has been described previously (19). The wild type of proHB-EGF was introduced into SKOV3 cells, and the transfected cells, called as SK-HB cells, were selected with 400 μ g/mL G418. R-181 cells were selected with 5 μ g/mL puromycin after transfection of the HB-EGF small interfering RNA (siRNA) vector into RMG1 cells. SK-HB and R-181 cells have previously been established (17). To construct the TGF α or amphiregulin (AR) small interfering RNA (siRNA) vector (pH1-PUR/siRNA TGF α or pH1-PUR/siRNA amphiregulin), we synthesized the following DNA oligonucleotides: TGF α , 5'-CCC GCCACGATTTCAAGACTTGTTC AAGAGAACAAGTCTTGAAATCGTGGCTTTTTGGAAA-3' and 5'-TTTCCAAAAGCCACGATTTCAAGACTTGTTCCTTTGAACAAGTCTTGAAATCGTGGCGGG-3'; amphiregulin, 5'-CCC GGCTGAGGAACGAAAGAAACTTCAAGAGAGTTTCTTTTCGTTCTCAGCCTTTTGGAAA-3' and 5'-TTTCCAAAAG GCTGAGGAACGAAAGAACTCTCTTGAAGTTTCTTTTCGTTCTCAGCCGGG-3' (20, 21). After amplification of the cDNA fragments by PCR, the fragments were cloned into the *Pst*I and *Xba*I sites of the pH1 RNA interference vector as described previously (17). Transfections of the constructs into RMG1 cells were done using LipofectAMINE 2000 reagent (Invitrogen Corp.) according to the manufacturer's instructions. Transfected cells with constitutive expression of the construct have been established as R-AR and R-TGF α . Each oligonucleotide siRNA target sequence for HB-EGF, TGF α , or amphiregulin, which was GTCCGTGACTTGCAAGAGG (NM_001945), GCATGTGTCTGCCATTCTG (NM_003236), or GCTGAGGAACGAAAGAAAC (M30704), was the same as the short hairpin RNA in the construct. The siRNAs for these EGFR ligands were individually transfected into cells using TransIT-TKO (Mirus) according to the manufacturers' instructions. The final concentration of each siRNA for transfection was 25 μ mol/L. All the experiments were done at 48 h after transfection of siRNAs into cells.

Cell Culture

RMG1, SKOV3, and the transfected cells were maintained in RPMI 1640 supplemented with 100 units/mL penicillin G, 100 μ g/mL streptomycin, and 10% fetal bovine serum (ICN Biomedicals).

Cell Spreading Assay

Cells were detached with trypsin-EDTA to remove extracellular matrix components, and then allowed to recover for 30 min in RPMI 1640 with 10% fetal bovine serum. After rinsing with serum-free medium, these cells were incubated with serum-free medium at 37°C for 30 min

and then seeded on coverslips, which had previously been coated with 10 $\mu\text{g}/\text{mL}$ fibronectin, collagen type I, or collagen type III at 37°C for 1 h, and then fixed in PBS with 4% paraformaldehyde and 5% sucrose. To further address the pharmacologic effects on cell adhesion, the cell spreading assay was done in the presence of the agents of interest in the serum-free medium. Cell spreading was analyzed by phase-contrast microscopy and calculated as the ratio of A/B , where A equals the number of spreading cells and B equals the number of total cells. Each experiment was done in triplicate, and the experiment was repeated at least twice.

Diphtheria Toxin Binding Assay

HB-EGF is a receptor for DT (15). After labeling a DT in use of ^{125}I , ^{125}I -labeled DT bound to HB-EGF was measured by scintillation counter to estimate the amount of HB-EGF. Cells (1×10^6) were treated as in the cell spreading assay and then seeded on 6-cm dishes coated with 10 $\mu\text{g}/\text{mL}$ poly-lysine or extracellular matrix (fibronectin, collagen type I, or collagen type III) in the presence of pharmacologic agents. Cells were incubated with serum-free RPMI 1640 at 37°C for 1 h to assume the complete adherence of the cells to the coated dishes. Binding of ^{125}I -labeled DT to cells was measured as described previously, and values of the specific binding were determined (22). To determine the amount of sHB-EGF, cells were seeded on 10-cm dishes (50–60% confluence) and incubated for 48 h. After that, each culture medium and the sHB-EGF bound to extracellular matrices on the cell surface supernatant were completely collected according to the procedures previously described (23). The mixed solution with culture medium and cell surface supernatant were incubated with heparin-sepharose to bind sHB-EGF to heparin-sepharose, and then binding of ^{125}I -labeled DT to heparin-sepharose was measured (22). Each experiment was done in triplicate, and the experiment was repeated at least twice.

Immunoblotting and Immunoprecipitation

Cells (1×10^6) were treated as described for the cell spreading assay and then seeded on 6-cm dishes coated with 10 $\mu\text{g}/\text{mL}$ poly-lysine or extracellular matrix (fibronectin, collagen type I, or collagen type III) in the presence of pharmacologic agents. Cells were incubated with serum-free RPMI 1640 at 37°C for 1 h to assume the complete adherence of the cells to the coated dishes. Cells were rinsed in PBS containing 1 mmol/L sodium orthovanadate and then lysed in immunoprecipitation assay buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0), 0.2 unit/mL aprotinin, 2 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ pepstatin A, 2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate]. Extracts and immunoprecipitants were then subjected to SDS-PAGE and immunoblotting analysis (24).

Cell Invasion Assay

The invasion assay was done using a chemotaxis chamber (Becton Dickinson and Co.) and transwell tissue culture plates (6.5 mm and 8- μm pore size). The bottom of

the chamber was coated with 10 $\mu\text{g}/\text{mL}$ Matrigel (Sigma). The uncoated sites were blocked with 10% bovine serum albumin. Cells (1×10^4) were introduced into each well and allowed to migrate for 6 h in RPMI 1640 with 10% fetal bovine serum. Cells were then fixed with methanol and stained with crystal violet. The migrated cells were quantified by counting the number of cells in five random $\times 200$ fields. Each experiment was done in triplicate, and the experiment was repeated at least twice.

Peritoneal Dissemination in Nude Mice

Cells were detached from plates with trypsin-EDTA and a total volume of 1 mL containing 1×10^7 cells suspended in serum-free RPMI 1640 was injected peritoneally into female BALB/c nu/nu mice at 4 to 5 wk of age (Charles River Laboratories). On the next day, CRM197 was dissolved in 20 mmol/L HEPES and 150 mmol/L NaCl (pH 7.2) and the amount of CRM197 (50 mg/kg) was injected i.p. into tumor-bearing mice in the abdomen each week for 6 wk. All injection-treated mice were fed for 7 wk after injection. At the end of 7 wk, the mice were sacrificed and each tumor burden in the peritoneal cavity was weighed. All experimental use of animals complied with the guidelines of Animal Care at Fukuoka University.

RNA Extraction and cDNA Synthesis

The human peritoneal tissues in patients, which were located near Douglas's cul-de-sac, were resected in 2 \times 2 cm size at the time of surgical operation and washed in PBS. The mesothelial cells in the peritoneum were curettaged. No cancer cells were microscopically found in these peritoneal tissues. All 20 patients in this study had undergone surgery at the Department of Obstetrics and Gynecology, Fukuoka University Hospital (Fukuoka Japan) between 2004 and 2006. The specimens were obtained from 10 cases with stage III ovarian cancer (as governed by the International Federation of Gynecology and Obstetrics) and 10 cases with normal ovaries being investigated for benign gynecologic disorders. An informed consent was previously acquired from all the patients in this study. RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was done with 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol.

Real-time Quantitative PCR and Reverse Transcription-PCR

The TaqMan quantitative PCR reaction was carried out using the sequences of the oligonucleotide primer pairs and TaqMan probes for each EGFR ligand and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (25). Experiments were carried out independently thrice. To examine the expression of Snail1, Slug, or GAPDH in ovarian cancer, reverse transcription-PCR analysis was done using primers specific for Snail1 (94°C for 15 s, 57°C for 30 s, 68°C for 60 s, 30 cycles; sense 5'-TGCGGAATCGGC-GACC-3', antisense 5'-CTAGAGAAGGCCTTCCCGCAG-3'), Slug (94°C for 15 s, 51°C for 30 s, 68°C for 60 s, 30 cycles; sense 5'-GCCTCCAAAAGCCAACTACAG-3',

antisense 5'-GTGTGCTACACAGCAGCC-3'), or GAPDH (94°C for 15 s, 60°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-ACCCAGAAGACTGTGGATGG-3', antisense 5'-TGCTGTAGCCAAATTCGTTG-3'). To assess the expression of HB-EGF, amphiregulin, TGF α , MMP9, or VEGF in ovarian cancer, reverse transcription-PCR was also done using primers specific for HB-EGF (94°C for 15 s, 60°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-GGTGGTGCTGAAGCTCTTTC-3', antisense 5'-CCCATGACACCTCTCTCCAT-3'), amphiregulin (94°C for 15 s, 59°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-CGGGAGCCGACTATGACTAC-3', antisense 5'-AACAGCAACAGCTGTGAGGA 3'), TGF α

(94°C for 15 s, 59°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-TCGCTCTGGGTATTGTGTTG-3', antisense 5'-TGTTTCTGAGTGGCAGCAAG-3'), MMP9 (94°C for 15 s, 56°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-CCTTCTACGCCACTACTGTG-3', antisense 5'-GCACTGCAGGATGCATAG-3'), or VEGF (94°C for 15 s, 58°C for 30 s, 68°C for 60 s, 30 cycles: sense 5'-AGCCAGCACATAGGAGAGA-3', antisense 5'-TTCTGCAGCTCTGTGTGAAGG-3'). Experiments were independently carried out thrice.

Statistical Analysis

Data were analyzed using the Mann-Whitney *U* test. *P* < 0.05 was considered statistically significant.

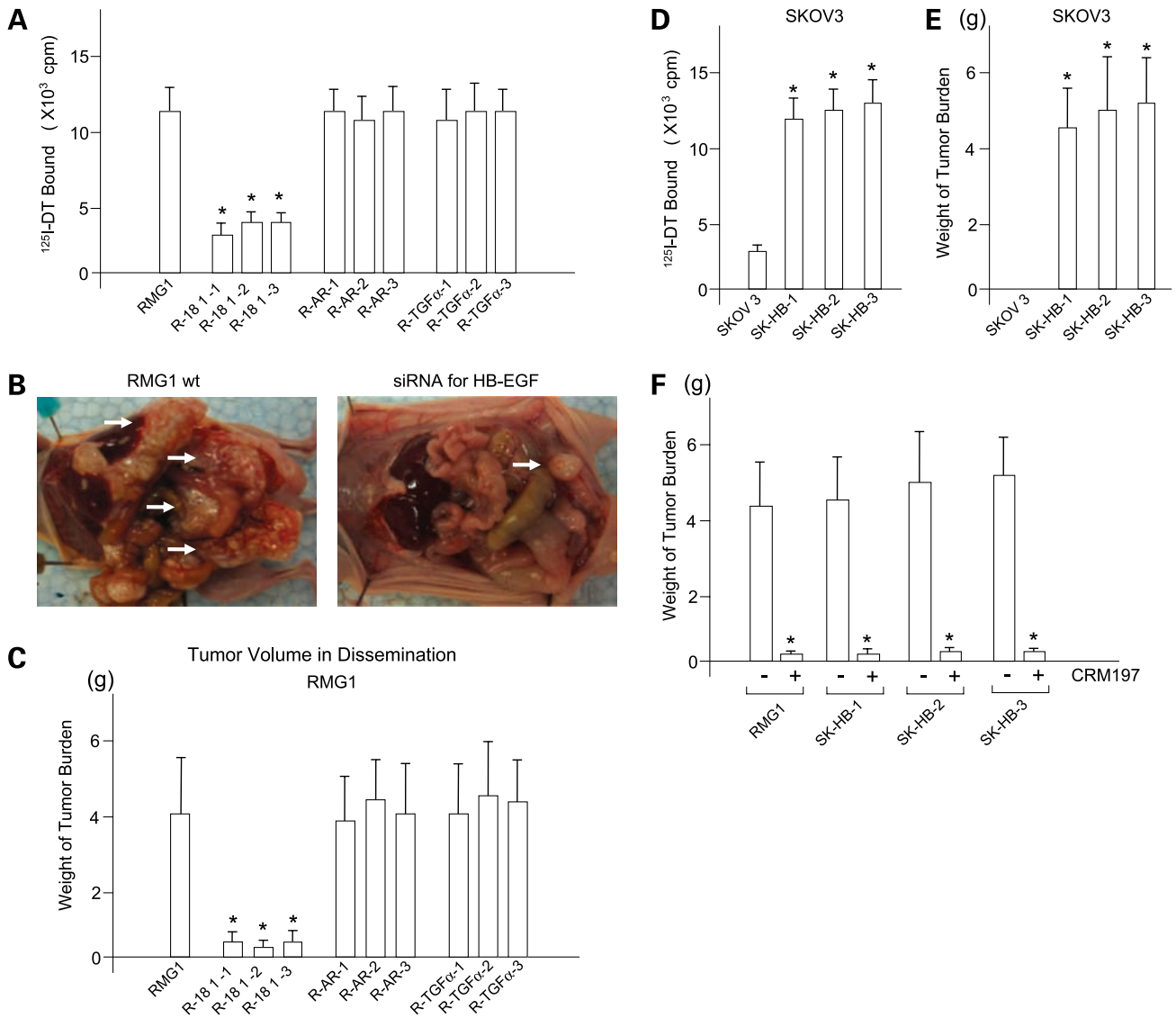


Figure 1. Transcoelomic metastasis mediated by HB-EGF in RMG1 and SKOV3 cells. **A**, expression of proHB-EGF on cell membrane in RMG1 cells and RMG1 cells transfected with siRNA for HB-EGF (R-181-1, R-181-2, and R-181-3), amphiregulin (R-AR-1, R-AR-2, and R-AR-3), or TGF α (R-TGF α -1, R-TGF α -2, and R-TGF α -3). **B**, tumor burden in RMG1 cells (left) or R-181-1 cells (right) at 7 wk after the injection of 1×10^7 cells. Arrows, disseminated lesions in the peritoneal cavity. **C**, total weight in tumor burden formed by RMG1, R-181, R-AR, and R-TGF α cells. **D**, expression of proHB-EGF on the cell membrane in SKOV3 cells and SKOV3 cells transfected with wild-type proHB-EGF (SK-HB-1, SK-HB-2, and SK-HB-3). **E**, total weight in tumor burden formed by SKOV3 cells and SK-HB cells. **F**, total weight in tumor burden formed by RMG1 cells and SK-HB cells with or without the administration of CRM197 (50 mg/kg) every week for 6 wk. Columns, mean of six data points; bars, SD. *, *P* < 0.01, versus the values of RMG1 or SK-HB cells.

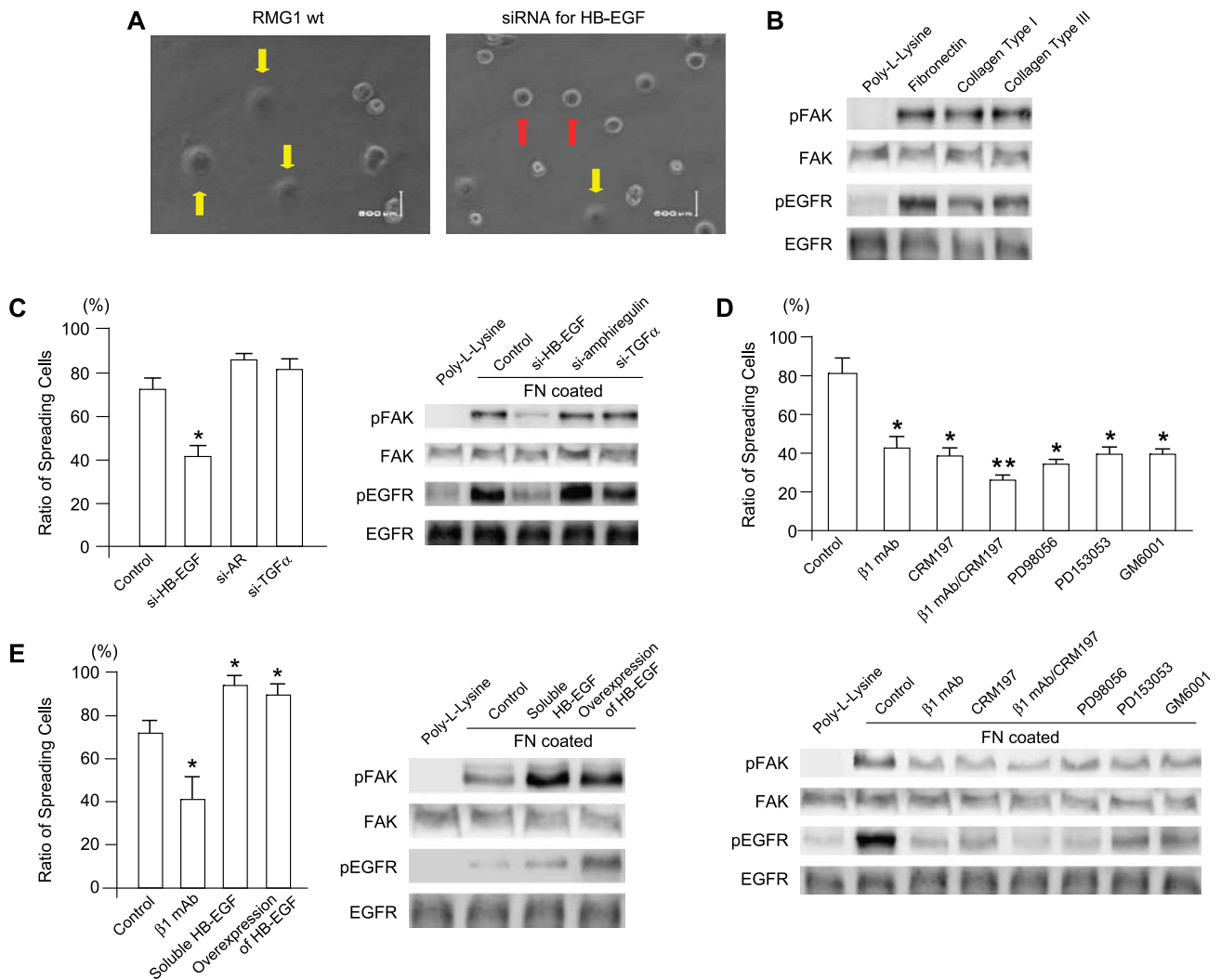


Figure 2. Cell adhesion properties on fibronectin, collagen type I, or collagen type III and signaling in RMG1 and SKOV3. **A**, adhesive cells of RMG1 cells (*left*) or RMG1 cells transfected with the siRNA oligonucleotide for HB-EGF (*right*) on fibronectin-coated dishes at 1 h after seeding of cells. RMG1 cells were well spread out peripherally (*yellow arrow*), whereas RMG1 cells transfected with siRNA for HB-EGF displayed the round shape (*red arrow*). **B**, activation of FAK and EGFR at 1 h after adhesion to poly-lysine, fibronectin, collagen type I, or collagen type III in RMG1 cells. **C**, percentage of spreading cells of the total cells and alterations in FAK and EGFR activation in RMG1 cells and RMG1 cells transfected with siRNA of HB-EGF, TGF α , or amphiregulin. **D**, percentage of spreading cells of the total cells and alterations in FAK and EGFR activation in the absence or presence of inhibitory antibody against integrin β_1 (10 μ g/mL), CRM197 (1 μ g/mL), PD98056 (50 μ mol/L), PD153053 (10 μ mol/L), or GM6001 (5 μ mol/L) in RMG1 cells. **E**, percentage of spreading cells of the total cells and alterations in FAK and EGFR activation in SKOV3 cells, SKOV3 cells transiently transfected with wild-type proHB-EGF, or SKOV3 cells with sHB-EGF (10 ng/mL). *Columns*, mean percentage of spreading cells for the total cells from six data points; *bars*, SD. *, $P < 0.01$, versus the values of RMG1 or SKOV3 cells as a control. **, $P < 0.01$, versus the values of RMG1 cells in the presence of inhibitory antibody against integrin β_1 , CRM197, PD98056, PD153053, or GM6001.

Results

Tumor Burdens in Peritoneal Dissemination Mediated by HB-EGF

To investigate the significance of HB-EGF involved in the transcoelomic metastasis of ovarian cancer, the tumor burden in the peritoneal cavity was measured at 7 weeks after the i.p. injection of RMG1, R-181, R-AR, or R-TGF α cells (1×10^7). The expression of proHB-EGF in R-181 cells significantly decreased, compared with those in the parental cells, R-AR cells, or R-TGF α cells (Fig. 1A). R-AR cells or R-TGF α cells had significantly low expression of

amphiregulin or TGF α , compared with that of the parental cells (Supplementary Fig. S1).⁴ The weight of the tumor burden formed by the R-181 cells was remarkably suppressed, compared with those from other cells, whereas no significant difference was found in the weight of the tumor burdens among the parental RMG1,

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

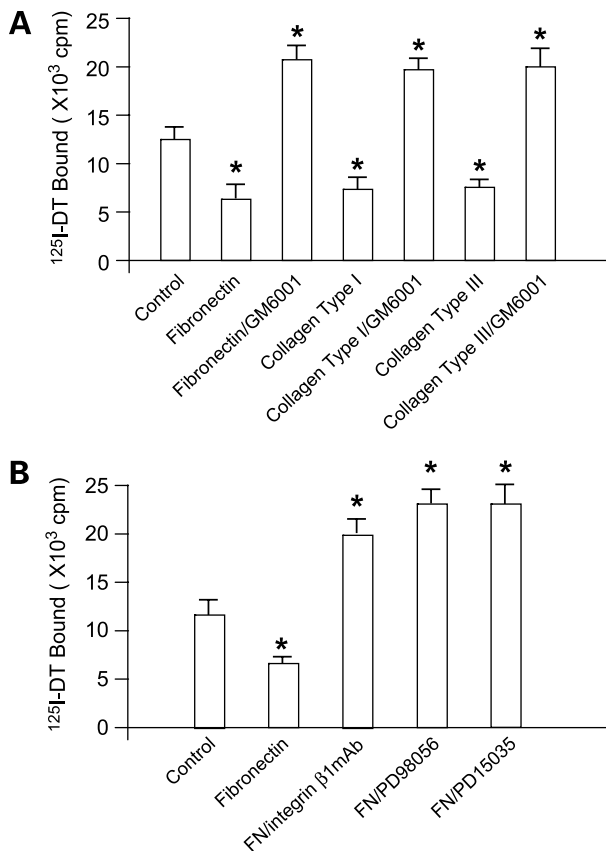


Figure 3. Ectodomain shedding of HB-EGF mediated by adhesion on fibronectin, collagen type I, or collagen type III. **A**, the expression of proHB-EGF on the cell membrane of RMG1 cells after the adhesion to poly-lysine, fibronectin, collagen type I, or collagen type III in the absence or presence of GM6001 (5 μ mol/L). **B**, the expression of proHB-EGF on the cell membrane of RMG1 cells after the adhesion to fibronectin in the absence or presence of inhibitory antibody against integrin β_1 (10 μ g/mL), PD98056 (50 μ mol/L), or PD153053 (10 μ mol/L). Columns, mean of six data points; bars, SD. *, $P < 0.01$, versus the values of RMG1 cells on the poly-lysine-coated dish as a control.

R-TGF α , and R-AR cells (Fig. 1B and C). SKOV3 cells had significantly less expression of HB-EGF than RMG1 cells (Fig. 1A and D). Transfected SKOV3 (SK-HB) cells had ~7-fold higher constitutive expression than SKOV3 cells (Fig. 1D). These SK-HB cells showed extensive tumor burdens although no peritoneal dissemination was seen after i.p. injection of SKOV3 cells (Fig. 1E). Taken together, these results suggest that the expression of HB-EGF facilitates peritoneal dissemination in ovarian cancer.

Moreover, the weight of the tumor burden was examined after the administration of CRM197, a specific inhibitor for HB-EGF. RMG1, SK-HB-1, SK-HB-2, and SK-HB-3 cells (1×10^7) were i.p. injected, and then on the next day, CRM197 (50 mg/kg) was administered i.p. every week for 6 wk. Each parental cell had a definite tumor formed in the peritoneal cavity, whereas those grown in the presence of CRM197 had a markedly suppressed tumor burden (Fig. 1F).

Cooperation in Cell Adhesion and HB-EGF Signaling

To test cell adhesion properties and signaling mediated by integrin on extracellular matrices from abdominal peritoneum, a cell spreading assay and alterations in signaling were done using RMG1 cells, RMG1 cells transfected with oligonucleotide siRNAs, SKOV3 cells with or without sHB-EGF, and SKOV3 cells transiently transfected with the vector harboring wild-type proHB-EGF. In RMG1 cells, significant adhesion was found on fibronectin, collagen type I, and collagen type III, accompanied by the activation of FAK as well as EGFR (Fig. 2B). In RMG1 and SKOV3 cells, HB-EGF was abundantly expressed, compared with that of amphiregulin or TGF α , using real-time PCR (Supplementary Fig. S2).⁴ The transfection of HB-EGF oligonucleotide siRNA into RMG1 cells blocked the cell spreading and signaling on fibronectin (Fig. 2C). These results suggest that HB-EGF, but not amphiregulin or TGF α , may play central roles in the activation of EGFR as well as FAK signal. The cell adhesion properties were also blocked by the antibody against integrin β_1 , CRM197 (an inhibitor for HB-EGF), PD153053 (an inhibitor for EGFR), PD98056 (an inhibitor for MEK1), or GM6001 (a protease inhibitor for the ectodomain shedding of HB-EGF; Fig. 2D). The combination of the inhibitory antibody against integrin β_1 with CRM197 significantly suppressed the cell adhesion and signaling, compared with the use of integrin β_1 antibody or CRM197 alone (Fig. 2D). When sHB-EGF was added or there was overexpression of wild-type proHB-EGF in SKOV3 cells, the cell spreading and activation of FAK and EGFR on fibronectin significantly increased, compared with SKOV3 cells alone (Fig. 2E). The data for fibronectin were consistent with the data for collagen type I or type III (data not shown). These results suggest that HB-EGF modulates cell adhesion properties as well as signaling mediated by integrin in ovarian cancer.

To evaluate the effect of cell adhesion on extracellular matrices in ectodomain shedding of HB-EGF, a DT binding assay was done using RMG1 cells. The expression of proHB-EGF on fibronectin, collagen type I, or collagen type III significantly decreased, compared with that on poly-L-lysine, and the reduction of proHB-EGF expression on extracellular matrices was inhibited by the use of metalloprotease (Fig. 3A). The inhibitory antibody against integrin β_1 as well as inhibitors for MEK1 and EGFR suppressed the ectodomain shedding of HB-EGF mediated by adhesion on fibronectin (Fig. 3B). These results suggest that cell adhesion on extracellular matrices induces the ectodomain shedding of HB-EGF as well.

Invasion and Angiogenesis Mediated by HB-EGF Expression

To elucidate invasiveness and angiogenesis dependent on HB-EGF expression, the invasive ability in the chemotaxis chamber and the expression of MMP9 and VEGF were examined using RMG1 cells, RMG1 cells transfected with oligonucleotide siRNAs, SKOV3 cells with or without sHB-EGF, and SKOV3 cells transiently transfected with the vector harboring wild-type proHB-EGF. The presence of inhibitors for HB-EGF, EGFR, and MEK1 or a protease

inhibitor for ectodomain shedding of HB-EGF suppressed the invasive properties in RMG1 cells, whereas little inhibition of the invasive ability in the chemotaxis chamber was observed when integrin function was blocked (Fig. 4A). The transfection of oligonucleotide siRNA for HB-EGF, not amphiregulin nor TGF α , into RMG1 cells significantly reduced the invasive ability in the chemotaxis chamber, compared with those in RMG1 cells (Fig. 4B). In addition to these results, a decreased expression of HB-EGF in RMG1 cells resulted in the suppression of MMP9 and

VEGF, whereas the inhibition of TGF α or amphiregulin did not alter the expression of VEGF in RMG1 cells (Fig. 4D). The mRNA expression of MMP9 or VEGF was significantly reduced by the transfection of siRNA for HB-EGF (Supplementary Fig. S3).⁴ In SKOV3 cells, overexpression of HB-EGF or the presence of sHB-EGF enhanced the invasive ability in the chemotaxis chamber and the expression of MMP9 and VEGF (Fig. 4C and E). These results suggest that HB-EGF activates invasive and angiogenic properties in ovarian cancer.

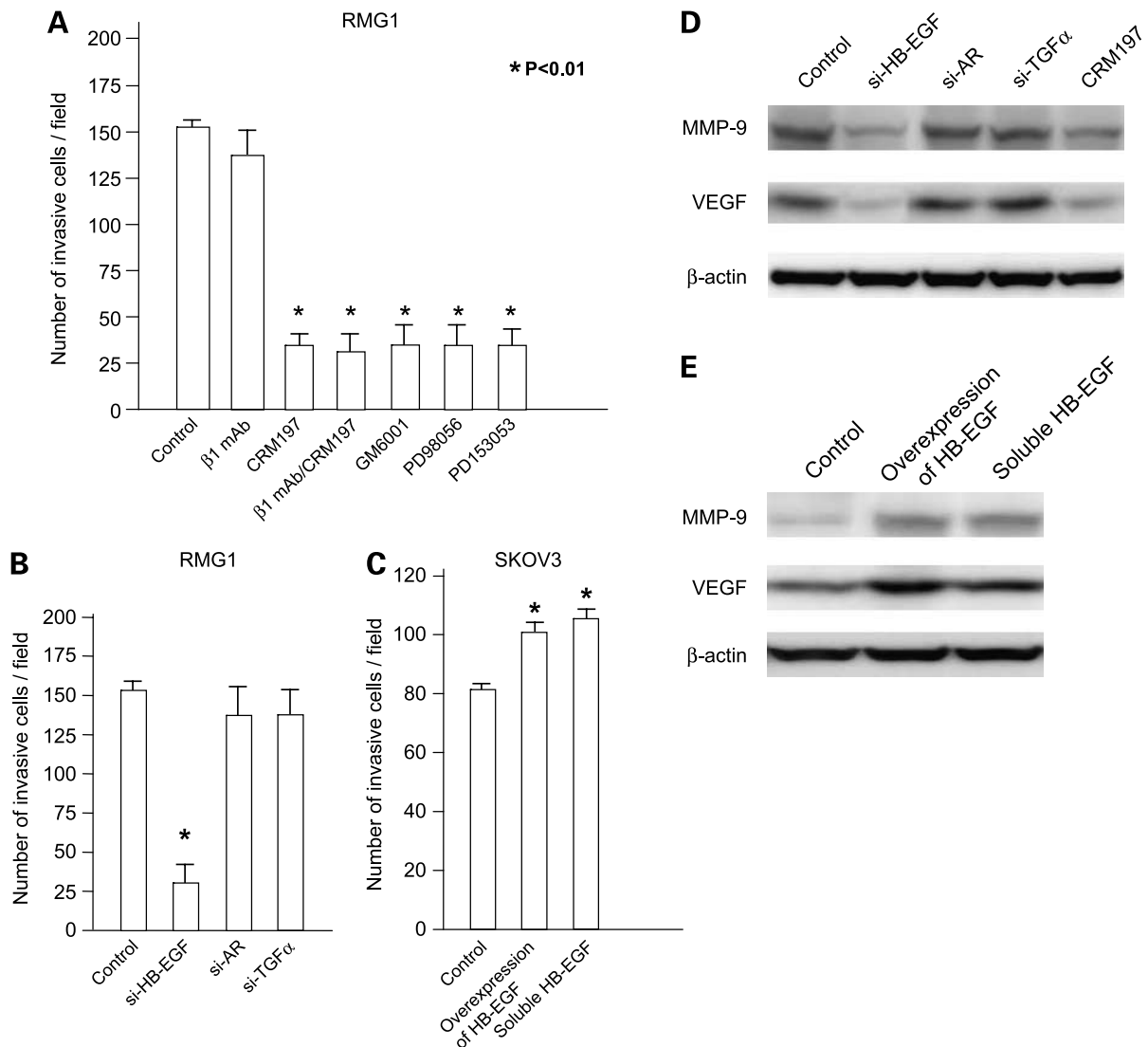


Figure 4. Alterations in the invasive or angiogenic ability. **A**, the number of invasive cells per field in the chemotaxis chamber in the absence or presence of inhibitory antibody against integrin β ₁ (10 μ g/mL), CRM197 (1 μ g/mL), PD98056 (50 μ mol/L), PD153053 (10 μ mol/L) or GM6001 (5 μ mol/L) in RMG1 cells, compared with that of the parental RMG1 cells (control, 100%). **B**, the number of invasive cells per field in the chemotaxis chamber for the transfected RMG1 cells with siRNA of HB-EGF, TGF α , or amphiregulin, compared with that of the parental RMG1 cells (control, 100%). **C**, the number of invasive cells per field in the chemotaxis chamber for the transfected SKOV3 cells with the wild type of proHB-EGF or SKOV3 cells in the presence of sHB-EGF (10 ng/mL), compared with that of the parental SKOV3 cells (control, 100%). **D** and **E**, the expression of MMP9, VEGF, IL-8, and β -actin in the parental RMG1 cells as a control; RMG1 cells transfected with siRNA of HB-EGF, TGF α , or amphiregulin; or RMG1 cells in the presence of CRM197 (1 μ g/mL; **D**, top lane); and the parental SKOV3 cells, the transfected SKOV3 cells with the wild type of proHB-EGF, or SKOV3 cells in the presence of sHB-EGF (10 ng/mL; **E**, bottom lane). Columns, mean of six data points; bars, SD. *, $P < 0.01$, versus the values of RMG1 or SKOV3 cells as a control.

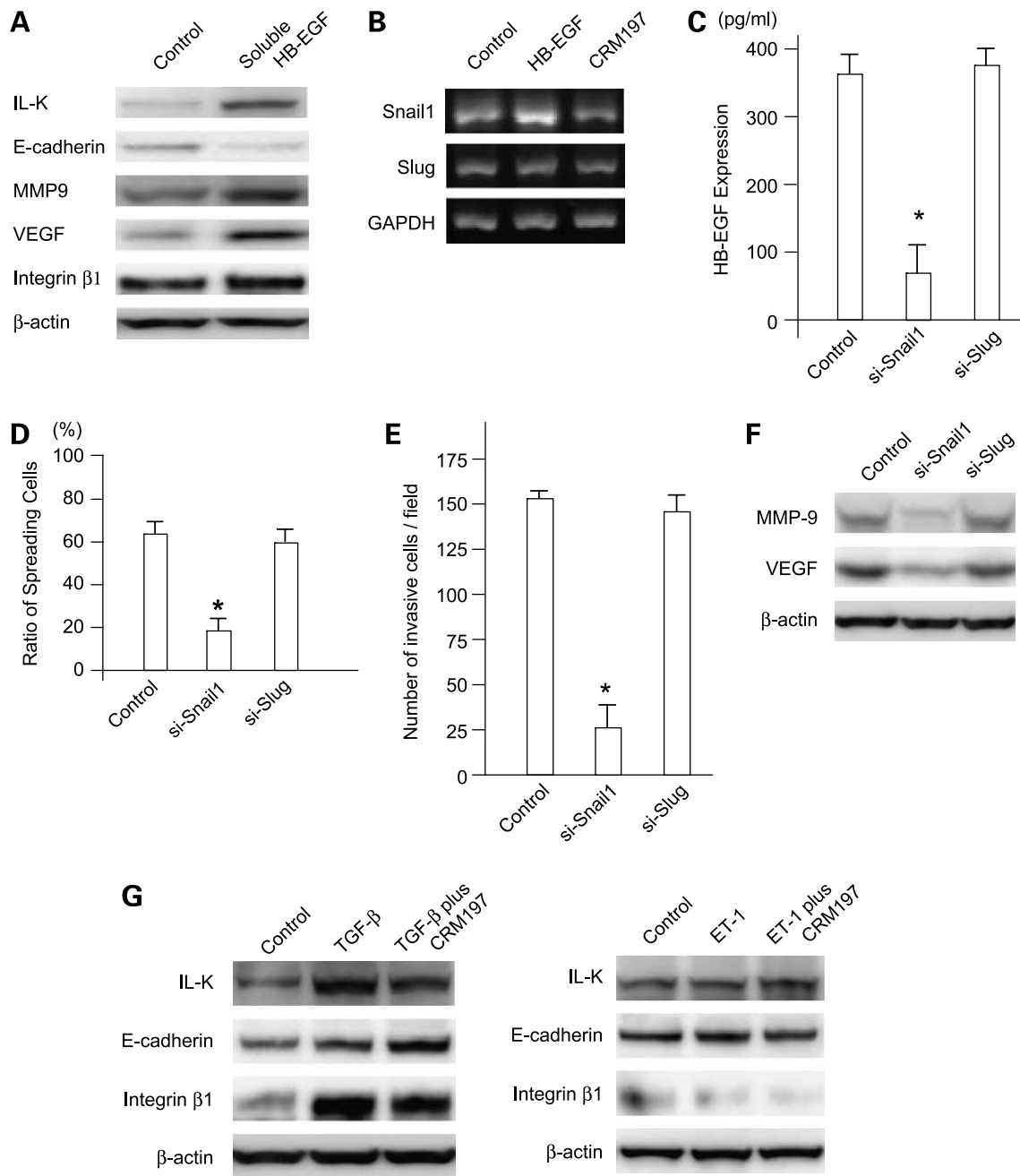


Figure 5. Association between EMT and HB-EGF in ovarian cancer. **A**, expression of EMT-related molecules at 6 h after the stimulation of HB-EGF (10ng/mL) in RMG1 cells. **B**, expression of Snail1 and Slug at 6 h after the stimulation of HB-EGF (10ng/mL) with or without CRM197 (1 μg/mL) in RMG1 cells. **C**, expression of sHB-EGF in the culture medium in RMG1 cells as a control and RMG1 cells transfected with the siRNA of Snail1 or Slug. **D**, percentage of spreading cells on fibronectin of the total cells in RMG1 cells as a control and in RMG1 cells transfected with siRNA of Snail1 or Slug. **E**, the number of invasive cells per field in the chemotaxis chamber in RMG1 cells transfected with the siRNA of Snail1 or Slug, compared with that of the parental RMG1 cells (control, 100%). **F**, expression of MMP9 and VEGF in RMG1 cells and RMG1 cells transfected with siRNA of Snail1 or Slug. **G**, expression of EMT-related molecules at 6 h after the stimulation of TGFβ (5 ng/mL) or ET-1 (100 nmol/L) with or without CRM197 (1 μg/mL) in RMG1 cells. *Columns*, mean of six data points; *bars*, SD. *, *P* < 0.01, versus the values of RMG1 cells as a control.

Relationship between HB-EGF and Snail Family Genes in Transcoelomic Metastasis

To assess the occurrence of EMT induced by HB-EGF, the expression of integrin-linked kinase and E-cadherin in

RMG1 cells was analyzed in the presence of sHB-EGF. HB-EGF augmented the expression of integrin-linked kinase, MMP9, VEGF, and integrin β₁ and reduced the expression of E-cadherin in RMG1 cells (Fig. 5A). In addition, HB-EGF

enhanced the expression of Snail1, not Slug (Fig. 5B), suggesting that HB-EGF induces EMT in ovarian cancer through Snail1. The expression of HB-EGF was significantly reduced by the transfection of siRNA for Snail1, but not for Slug (Supplementary Fig. S4).⁴ To elucidate whether Snail1 or Slug is associated with a malignant phenotype mediated by HB-EGF, the expression of HB-EGF, by a cell spreading and invasion assay, was compared between RMG1 cells and RMG1 cells transfected with oligonucleotide siRNA of Snail1 or Slug. The suppression of Snail1 induced a decreased expression of HB-EGF in the DT binding assay, compared with that in RMG1 cells or RMG1 cells transfected with oligonucleotide siRNA for Slug (Fig. 5C). The mRNA expression of MMP9 or VEGF was significantly reduced by the transfection of siRNA for Snail1, but not for Slug (Supplementary Fig. S4).⁴ The cell adhesion on fibronectin as well as the invasion property was also significantly blocked by the suppression of Snail1 expression (Fig. 5D and E). To further address the association between HB-EGF and other inducers of EMT including TGF β and endothelin-1, the expression of integrin-linked kinase and E-cadherin in RMG1 cells was examined in the presence of TGF β and endothelin-1 with or without CRM197. The expression of integrin-linked kinase and integrin β_1 induced by TGF β was in part suppressed by CRM197, whereas no significant difference in expression of integrin-linked kinase, E-cadherin, and integrin β_1 was found with the stimulation of endothelin-1 (Fig. 5F), suggesting that EMT mediated by TGF β may be associated with HB-EGF. According to these results, HB-EGF may play a pivotal role in the acquisition of aggressive behavior in ovarian cancer through EMT.

Expression of EGFR Ligands in Human Peritoneal Tissues

To investigate the facility of peritoneal cells in transcoelomic metastasis, the expression of EGFR ligands in peritoneal tissues was examined in patients with normal ovaries or ovarian cancer. In patients with advanced ovarian cancer, HB-EGF expression was significantly elevated

compared with other EGFR ligands (Fig. 6). The expression of HB-EGF, amphiregulin, or epiregulin in patients with advanced ovarian cancer was significantly elevated, compared with that in patients with normal ovaries (Fig. 6). Each expression index of EGF or epigen was <0.001 in patients with advanced ovarian cancer and normal ovaries. These results indicated that HB-EGF was expressed as the predominant molecule among EGFR ligands in the mesothelial cells of patients with advanced ovarian cancer.

Discussion

In this study, we showed that HB-EGF enhances cell adhesion, invasion, and angiogenesis in ovarian cancer, resulting in the peritoneal dissemination through EMT. Furthermore, HB-EGF was shown to promote cell adhesion on extracellular matrices via integrin β_1 and enhance invasion and angiogenesis. Previously, we have shown that HB-EGF is enriched enough to help cancer cells survive in the peritoneal fluid of patients with ovarian cancer, leading to the recognition of HB-EGF as one of the ovarian activating factors. Accordingly, HB-EGF fulfills the key roles involved in each step of peritoneal dissemination, indicating that HB-EGF is recognized as a mutual target for advanced ovarian cancer therapy.

In this study, cell adhesion to extracellular matrices evoked the activation of FAK and EGFR. HB-EGF expression was predominantly abundant in ovarian cancer compared with amphiregulin or TGF α expression. The suppression of HB-EGF expression attenuated the tyrosine phosphorylation of FAK as well as EGFR, although alterations in amphiregulin or TGF α expression had little effects on cell adhesion or signalings. Moreover, the blockade of integrin function also reduced the activation of FAK and EGFR. According to these lines of evidence, we hypothesize as follows: Cells bind to extracellular matrices via integrin function, and the aggregation of integrin induces the accumulation of FAK. The accumulation of FAK evokes autophosphorylation of FAK, resulting in the activation of ERK. The activated ERK provokes the

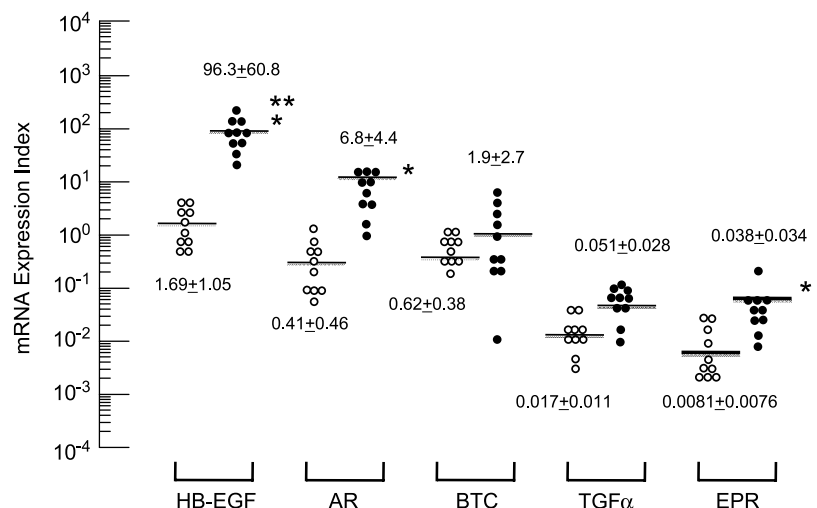


Figure 6. Expression of EGFR ligands in mesothelial cells of patients with normal ovaries and ovarian cancer. Closed or open circle, the value of expression of EGFR ligands in a patient with advanced ovarian cancer or normal ovaries. AR, amphiregulin; BTC, betacellulin; EPR, epiregulin. Each value represents the mean and SD of 10 patients. A bar indicates the mean value. *, $P < 0.05$, versus the values in patients with normal ovaries. **, $P < 0.05$, versus the values of the expression of other EGFR ligands.

cleavage of HB-EGF, leading to tyrosine phosphorylation of EGFR. On the other hand, the ectodomain shedding of proHB-EGF activates EGFR and ERK, contributing to FAK activation and subsequent aggregation of integrins at cell adhesion sites. In ovarian cancer, the autocrine loop of EGFR signal is formed by HB-EGF, but not by amphiregulin or TGF α . In principle, FAK is an important receptor-proximal link between growth factor receptor and integrin signaling pathways (26). Therefore, our study suggests that HB-EGF and integrin may interplay signalings through the activation of FAK and EGFR in ovarian cancer.

In epithelial cells, HB-EGF forms a complex with CD9, integrin α_3 , and heparan sulfate proteoglycan at cell-cell contact sites (27). EGFR also binds to integrin α_2 at cell-cell contact sites (28). Losses of CD9 and integrin α_3 were predominantly found in human cancers, whereas various kinds of heparan sulfate proteoglycan, HB-EGF, and EGFR are highly expressed on the surface of the cell membrane in cancer cells (29–31). Because HB-EGF contains the heparan sulfate proteoglycan, it is through this heparin binding motif that it can more readily accumulate on the cell surface and activate the EGFR pathway, compared with other EGFR ligands without the heparin binding motif (32). Additionally, through the complex formation of HB-EGF, matrix metalloprotease, and CD44, up-regulation of HB-EGF is also involved in the CD44 function activity of the female tract, leading to the enhancement of the adhesive properties of the peritoneum (33). CD44, which is a facultative proteoglycan and functions as a hyaluronan receptor, has been recognized as the other key molecule that cancer cells use to adhere to the peritoneal mesothelium via interaction with hyaluronan (34, 35). HB-EGF and its associated molecules, such as CD44 and hyaluronan, also induce EMT (36, 37). In particular, several growth factors, including TGF β , EGFR ligands, endothelin-1, etc., have been regarded as a direct inducer of EMT in ovarian cancer (38). Thus, HB-EGF as well as its associated molecules may participate in promoting transcoelomic metastasis in ovarian cancer.

The ovarian surface epithelium, which is the modified pelvic mesothelium that covers the ovary, is of major importance in gynecologic pathology because it is thought to be the source of epithelial ovarian cancer (39). Surface epithelial cells are continuous with the mesothelium of the ovarian ligament and the peritoneum. Therefore, ovarian surface epithelial cells as well as mesothelial cells in the peritoneum are commonly referred to as coelomic epithelial cells, which possess the characteristics of both epithelial and mesenchymal cells. In the process of repair and wound healing, coelomic epithelial cells can convert to a mesenchymal, fibroblast-like phenotype, a process known as epithelial-mesenchymal transition (EMT), and also can produce extracellular matrices including fibronectin, collagen type I, collagen type III, and other molecules associated with EMT (40, 41). EGF induces EMT in human ovarian surface epithelium, accompanied with enhanced expression and activation of MMP9, ERK, and integrin-linked kinase (42). The mesothelial cells involved in the peritoneal

dissemination of ovarian cancer also predominantly express HB-EGF. Previously, we reported that ovarian cancer cells have an abundant expression of HB-EGF (16). According to these pieces of evidence, HB-EGF stimulates the mesothelial and ovarian cancer cells to convert into mesenchymal cells and to produce VEGF and extracellular matrices, resulting in enhanced dissemination of cancer cells in the abdominal cavity.

Aberrant activation of the EGFR signal including overexpression of EGFR and abnormal stimulation by autocrine growth factor loops has been involved in cancer progression (43). The progressive growth and dissemination of solid tumors, such as ovarian cancer, is dependent on the process of angiogenesis, which is regulated by the equilibrium between proangiogenic and antiangiogenic molecules (44–46). Thus, EGFR and VEGF are candidate molecules as therapeutic targets for the peritoneal dissemination of cancer. Of note is that antiangiogenic gene therapy does not require a direct and selective transduction of target genes into cancer cells, but rather transduction around the tumor to create an antiangiogenic environment. Accordingly, it is plausible that antiangiogenic agents may synergize with cytotoxic drugs and radiation to improve therapeutic efficacy. To date, a number of new strategies of anti-VEGF therapy with bevacizumab are in development in patients with recurrent cancer, including novel combinations with other biological therapeutics targeting EGF receptors and conventional cytotoxic agents (47, 48). HB-EGF, which directly stimulates the production of VEGF in vascular endothelial cells, activates both ovarian cancer cells and mesothelial cells to produce VEGF expression (49). Therefore, it is plausible that anti-HB-EGF therapy may consist of anti-VEGF therapy and anti-EGFR therapy in patients with advanced ovarian cancer.

CRM197, a specific inhibitor for HB-EGF, blocked the properties associated with cell adhesion, invasion, and angiogenesis, and reduced the volume of the tumor burden in nude mice. CRM197 has previously been available for patients with cancer in a clinical research study (50). Under the approval of the ethics committee, a phase I study on the use of CRM197 has been done at Fukuoka University for patients with advanced ovarian cancer. Further clinical trials on the use of CRM197 would allow us to explore this novel therapeutic agent for advanced ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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